



Sequential origin in the high performance properties of orb spider dragline silk

SUBJECT AREAS:

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Major ampullate (MA) dragline silk supports spider orb webs, combining strength and extensibility in the toughest biomaterial. MA silk evolved ~376 MYA and identifying how evolutionary changes in proteins influenced silk mechanics is crucial for biomimetics, but is hindered by high spinning plasticity. We use supercontraction to remove that variation and characterize MA silk across the spider phylogeny. We show that mechanical performance is conserved within, but divergent among, major lineages, evolving in correlation with discrete changes in proteins. Early MA silk tensile strength improved rapidly with the origin of GGX amino acid motifs and increased repetitiveness. Tensile strength then maximized in basal entelegyne spiders, ~230 MYA. Toughness subsequently improved through increased extensibility within orb spiders, coupled with the origin of a novel protein (MaSp2). Key changes in MA silk proteins therefore correlate with the sequential evolution high performance orb spider silk and could aid design of biomimetic fibers.

The major ampullate (MA) silk utilized by orbicularian spiders in the construction of their iconic orb webs is the toughest biological material known¹. Coupled with the silk's production at ambient temperatures in living tissue and its biocompatibility², orb spider MA silk is therefore an important model for biomimicry. While the ecological function of MA silk as the energy-absorbing backbone of aerial orb webs likely acted as a critical agent of natural selection for its remarkable toughness³, MA silk evolved much earlier in the history of spider diversification. Ancestrally, MA silk was utilized primarily in terrestrial webs that simply entangled pedestrian insects or acted as lifelines⁴. The innovative behavioral shift of utilizing MA silk to construct discrete aerial frameworks for the webs of orb-weaving spiders⁴ was accompanied by significant changes in the genes encoding MA silk proteins⁵. Phylogenetic comparison of the mechanical performance of major ampullate silk suggests that at least some aspects of its impressive mechanical performance predate the origin of the orb web by hundreds of millions of years^{3,6,7}, providing substantial opportunity to understand how the impressive material properties of orb spider MA silk arose. However, MA silk properties are poorly investigated outside of orb web spiders. Moreover, some spiders exert substantial control over structure as silk is spun resulting in large intra-specific and even intra-individual variability in silk performance⁸, severely obscuring relationships between the evolution of spider silk genes and silk performance.

We overcome this limitation by exposing MA silk fibers to water to supercontract them to their “ground state”, where mechanical performance is independent of previous loading history^{9,10}, to understand the sequence of evolution of orb spider's MA silk toughness over its ~376 million year history. Spiders control the properties of their MA silk by applying shear force to silk as it emerges from their spinnerets¹¹. This shear force helps to determine the overall alignment of silk proteins, which is then “frozen” into place by hydrogen bonding^{9,12}. Silk processing allows spiders to tailor MA silk performance to ecological function, but also obscures inherent variability determined by protein composition *per se*. During supercontraction¹³, water infiltrates silk and disrupts hydrogen bonding, allowing the fiber to return to its “ground state”. This allows comparison of the intrinsic properties of silk performance^{9,14,15} and is particularly important because supercontraction itself varies substantially among spider taxa¹⁶.

Results

The material properties of MA silk are shown in Figure 1 for representatives of all major groups of spiders. We use the aciniform silk spun by Mygalomorphae (tarantulas and their relatives), the sister taxa to Araneomorphae, as

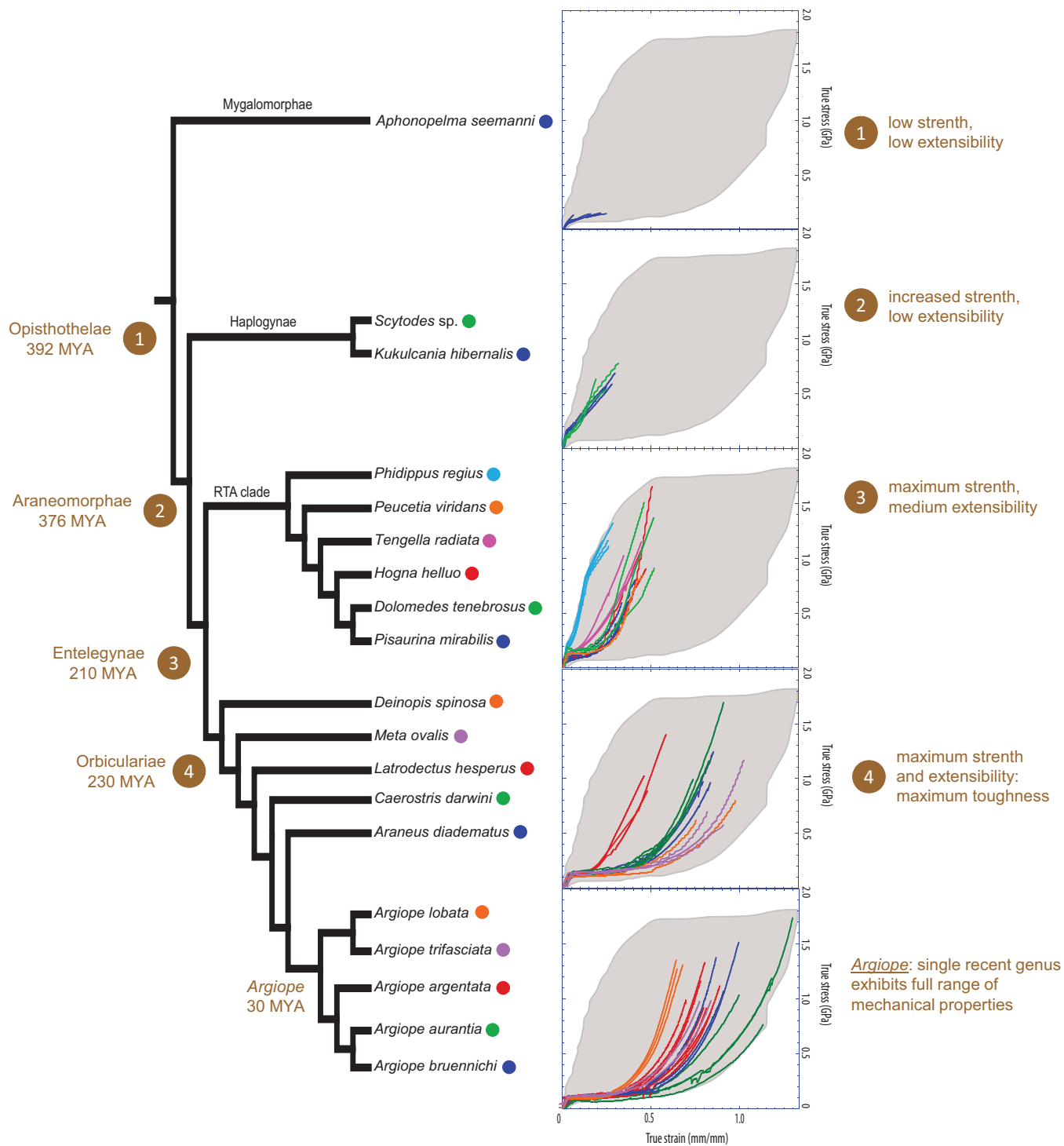


Figure 1 | Phylogenetic variation in major ampullate (MA) silk performance. Gray denotes the performance space occupied by MA spider silk. We identify four key events in MA evolution that explain the extraordinary toughness and plasticity of modern orb spider silk. Details are given in the text.

an outgroup¹⁷ to infer the likely ancestral properties of early MA silk. Among orb spiders, MA silk is a composite of two types of proteins (major ampullate spidroins: MaSps) dominated by four types of highly repetitive amino acid sequences called motifs¹⁸. Tarantula silk contains low frequencies of two of these motifs – glycine-alanine (GA) and poly-alanine (poly-A) – which in MA silk form β -sheets that stack together into nanocrystals, interlocking silk molecules. The existence of nanocrystals in tarantula silk is untested, but GA and poly-A motifs form nanocrystals in the MA silk of all studied species¹⁹. However, the amino acid sequences of mygalomorph silk pro-

teins are highly heterogeneous^{5,17,20}, therefore containing only few GA and poly-A motifs. This heterogeneity of amino acid sequences correlates with relatively poor performance of their silk. Elastic modulus ($E=2.5\pm0.6$ GPa), tensile strength ($\sigma_u=140\pm10$ MPa) and strain at breaking ($\epsilon_u=0.17\pm0.08$) are lower than other spider taxa, leading to approximately ten fold lower work to fracture ($W_f=16\pm9$ MJ/m³), compared to MA silks. Tarantula silk also does not supercontract (Fig. S1). Thus, ancestral MA silk likely was inferior in performance and spinning plasticity to all modern araneomorph spiders.



MA silk performance improved rapidly among basal lineages of araneomorph spiders that diverged from one another ~376MYA, correlating with three key changes in silk proteins. Haplogyne spiders are among the most basal taxa to produce MA silk, but their amino acid sequences lack many key features found in orb spider MA silks. Both poly-A and GA motifs are present, and appear more uniform compared with those observed in Mygalomorphae, but the overall amino acid sequences of haplogyne MA silk is still quite heterogeneous (Fig. S1). Haplogyne MA silk cDNAs notably contain a few isolated glycine-glycine-X motifs (GGX; where X is a small subset of amino acids), which are hypothesized to be plasticized by water during supercontraction^{13,21}. Glycine favors ordered backbone turns that are stabilized by hydrogen bonding among amino acid residues when dry, but promotes mobility and disorder of protein backbones when water disrupts those hydrogen bonds²². Thus, hydration of glycine-rich residues relaxes MA silk through the glass transition and accounts for one component of the supercontraction response of orb spider MA silk²³. However, full mobilization of the amorphous fraction of MA silk is only achieved by an interaction between glycine and proline residues²³. Because haplogyne silks contain relatively few glycine residues, and almost no proline, they show modest supercontraction of approximately 10% or less (Fig. S1), and reach an elastic modulus of $E=5.5$ to 8.6 GPa (for *Scytodes* and *Kukulcania* respectively), which is higher than the ground state in evolutionarily derived lineages. Tensile strength and strain at breaking (maximum values, *Scytodes*, $\sigma_u=640\pm10$ MPa, *Kukulcania* $\sigma_u=0.27\pm0.06$) are still much lower than derived taxa, but work to fracture is higher than that of Kevlar or ultra-high-molecular-weight-polyethylene (*Scytodes*, $W_f=90\pm40$ MJ/m³, Kevlar, $W_f=50$ MJ/m³). Thus, remarkably high performance silk fibers evolved early in the history of spiders, at least 130MY before the origin of aerial orb webs (Figs S2–S3).

The next innovation in MA silk genes occurred at the base of Entelegynae, prior to the divergence of orb spiders and RTA clade. No new amino acid motifs appear in their common ancestor (Fig. S1), but the GGX motif is significantly more frequent and repetitive compared to basal taxa. This increases the elastomeric properties of the proteins²². MA silk continues to increase in extensibility ($\epsilon_u=0.46$) within Entelegynae (Fig. S3) and reaches a maximum tensile strength that is similar between RTA clade and Orbiculariae ($\sigma_u=980\pm20$ and 1140 ± 30 MPa respectively) (Fig. S2). This combination yields a large work to fracture ($W_f=149$ MJ/m³) at the base of the Entelegynae at least 230 MYA (Fig. S3). Within Entelegynae, the RTA clade silk still lacks some key features of orb spider silk. Moreover, RTA clade MA silks show remarkably similar tensile properties among species, despite evolutionary divergence for over 150 MYA. *Phidippus* silk is notably stiffer, which may correlate with the unusual hunting ecology of the family Salticidae.

Discussion

The unrivaled toughness of orb spider MA silk coincides with the appearance of a novel MaSp2 protein, at the base of Orbiculariae. MaSp2 contains a high content of larger side chain amino acids and, in particular proline, which is incorporated into a novel glycine-proline-glycine-glycine-X motif (where X is a small subset of amino acids; GPGGX). Proline generally destabilizes secondary structures in proteins, favoring amorphous protein networks²². The GPGGX motif kinks the backbone of the peptide into an extensible β -spiral²⁴, which explains in part the up to five fold greater extensibility of the MA silk of orb spiders compared to RTA clade ($\epsilon_u = 0.8\pm0.2$ vs 0.39 ± 0.09). This high extensibility explains why work to fracture is ~100% greater in orb spiders compared to RTA clade ($W_f = 290\pm90$ vs 160 ± 50 MJ/m³). The combination of high toughness and extensibility in the MA silk of orb spiders plays a critical role in how webs resist breaking under the impact of flying prey^{25,26}.

Proline-rich MaSp2 imparted MA silk with a new flexibility at two very different levels. First, Orbiculariae MA silk extensibility in its ground state varies almost three-fold among taxa ($\epsilon_u = 0.51$ to 1.3). The proline content of MA silk in orb spiders correlates closely with interspecific variation in compliance and extensibility^{10,27}. In contrast, the tensile behavior of haplogyne and RTA clade silks are homogeneous within each clade. This suggests that shifts in the expression of MaSp1 versus MaSp2 provide a highly evolvable mechanism tailoring the functional properties of MA silk in different species of orb spiders. This is particularly evidenced by *Argiope*. This genus is ~30MY old, yet the performance of MA silk varies to almost the same degree as exhibited by the rest of the 210MY old Orbiculariae (Fig. 1). This high evolvability results in the MA silk of orb spiders occupying three quarters of the total performance space delimited by all other spider species.

Second, MaSp2 facilitates performance plasticity within individual orb spiders¹⁰. Supercontraction occurs in part as the GPGGX motif interacts strongly with water²³, mobilizing the amorphous fraction of the silk. By stretching MA fibers in water, the amorphous fraction is increasingly aligned and this structure can be held in place by hydrogen bonds when the silk dries. This stiffens the silk and allows any particular stress-strain curve in the range of accessible properties to be reached in a predictable and reproducible way. This 'wet stretching' can therefore make the MA silk from a single orb spider occupy almost any region of the performance space in Fig. 1 from its ground state to the left of the performance space⁹. During natural spinning, shear forces in the duct of the MA gland align silk molecules in still wet fibers, pulling them out of their ground state. Thus, spiders whose silk supercontracts strongly can access a greater range of performance for MA silk^{9,10}.

Our results draw a coherent picture of spider dragline silk evolution, relating major innovations in MaSps to changes in silk functional properties. The origin of the orb web is a singular event in the evolutionary history of spiders that played a dominant role in the evolution of silk, but we reveal that the history of MA silk is significantly more complex. MA silk performance is characterized by stability within lineages, punctuated by evolutionary changes that correlate with innovations in molecular composition. Basal MA silk shows poor mechanical properties compared to derived taxa. Increased homogenization and repetitiveness of GA and poly-A motifs, as well as the origin of a new GGX motif, in MA silk proteins correlate with increases in strength and stiffness that pre-date the origin of orb webs. However, the extreme toughness of orb spider MA silk is only reached after the evolution of a novel protein, MaSp2 that greatly improved extensibility. In addition to facilitating the function of aerial orb webs, MaSp2 facilitates inter- and intra-specific variation in the mechanical performance of MA silk. The discovery of these evolutionary correlations between the molecular composition and tensile properties of MA silk should facilitate constructing bioinspired fibers that mimic the outstanding properties of natural orb spider dragline silk²⁸.

Methods

Taxon selection, phylogenetic relationships, and collection of silk. Taxa span the phylogenetic diversity of MA silk producing spiders, with major lineages represented by species with diverse ecologies. Brief descriptions follow.

Mygalomorphae lack major ampullate glands but are sister to araneomorph spiders so that the undifferentiated sheets of silk that they spin provide the best available comparison to major ampullate silk. Mygalomorphae diverged from Araneomorphae at least 390MY ago²⁹. We tested silk from *Aphonopelma seemani*, purchased from the pet trade (Tarantulaspiders.com).

Araneomorphae "true spiders" consist of two large recent evolutionary radiations, RTA clade and Orbiculariae, and several basal clades, here represented by Haplogynae. All produce MA silk from a pair of glands on their anterior lateral spinnerets.

Haplogynae are ecologically diverse and diverged from other araneomorphs at least 375 MYA³⁰. Many use MA silk in the construction of terrestrial or aerial webs. The spitting spider *Scytodes* is a wandering hunter that primarily trails MA silk as a dragline while the southern house spider *Kukulcania hibernalis* uses MA silk in the



construction of prey capture webs. These webs are loosely affixed to the substrate and entangle walking prey. Both species were collected in Florida, USA.

RTA clade: Five families are included here. The wolf spiders Lycosidae (*Hogna helluo*) and the fishing spiders Pisauridae (*Dolomedes tenebrosus* and *Pisaurina mira*) are terrestrial ambush predators. The jumping spiders Salticidae (*Phidippus regius*) and the lynx spiders Oxyopidae (*Peucetia viridans*) are wandering hunters that crawl extensively over vegetation while searching for prey, often using MA silk as a lifeline or to abseil. Tenggellidae (*Tengella radiata*) spins an aerial prey capture web against trees that consists of a sheet held in place by a three-dimensional network of MA silk. *Dolomedes* and *Pisaurina* were collected in Bath, OH while *Phidippus* and *Peucetia* were obtained from the southwestern US (Spiderpharm.com). *Tengella radiata* was collected in La Selva, Costa Rica.

Orbiculariae: Orb webs evolved ~210MYA^{31,32} and are characterized by a framework of MA silk that includes radial lines supporting an adhesive capture spiral. These webs capture flying insects by first dissipating the kinetic flight energy and then adhering to the insects. The construction of the MA frame, which allows orbs to be suspended in the air column and defines the shapes of webs independently from the surrounding microhabitat, is likely a key innovation that facilitated the subsequent evolution of new web types⁴. In particular, many spiders within the orbiculariae no longer spin orb webs, and now produce cobwebs or aerial sheetwebs⁴. *Argiope* represents a relatively “standard” orb web. *Nephila* spins a highly asymmetric orb while *Meta ovalis* occupies humid cave environments. *Caerostris darwini* is exceptional in spinning gigantic orb webs across rivers in Madagascar using MA silk that is significantly tougher than other orb spiders¹. *Deinopis spinosa* utilizes cribellate capture threads, representative of the earliest orbicularian spiders that evolved orb web spinning behaviors prior to the evolution of viscid glue (i.e. their capture spirals use dry adhesive silk similar to basal taxa in the RTA clade and Haplogynae). Finally, the western black widow *Latrodectus hesperus* represents Theridiidae, which transformed the orb architecture into three-dimensional cobwebs. *Meta* were collected in Summit Co., Ohio. *Nephila* and *Deinopis* were collected in Gainesville, FL. *Caerostris darwini* was collected from Andasibe-Mantadia National Park, Madagascar. *Argiope* were collected in various US and European localities.

Phylogenetic relationships. No single phylogeny includes all species in our study. Instead, we used five studies to reconstruct different parts of the phylogeny. Deeper level relationships were taken from Blackledge et al.⁴ to define the major clades of interest. While apical relationships within RTA clade were not a focus of this study they were taken from Griswold et al.³³. Relationships within Orbiculariae were from Sensenig et al.³⁴. Finally, the topology among *Argiope* comes from Elices et al.³⁵ and Cheng et al.³⁶. Ancestral values for basal clades were reconstructed using Mesquite v2.75 under squared change parsimony³⁷.

Collection and characterization of silk. Silk was collected from spiders using forcible silking³⁸. Spiders were restrained on petri dishes, but conscious, and silk was drawn from the spinnerets at 2 cm/s. The spinnerets were visualized under a stereomicroscope to ensure that only threads originating from the major ampullate gland were collected. Silk was mounted across 15mm gaps on cardboard holders and secured with cyanoacrylate.

Maximum supercontraction and testing of silk. Two adjacent samples were cut from each fiber. One sample was used for tensile testing and mounted on an aluminium foil frame. The other sample was used for measuring the cross sectional area of the fiber. The gauge length of the samples prepared for testing was determined with a profile projector (resolution $\pm 10 \mu\text{m}$), and it was approximately 10 mm for all fibers.

The samples were mounted in an Instron 4411 tensile testing machine and the length at which the fiber was taut, but not subjected to load, was determined. The fiber slackened and, under this condition, immersed in water³⁹. After removing from the water, the fiber was allowed to dry overnight and then we checked that the fiber remained slack before starting the tensile test. Force-displacement curves were obtained by tensile testing. Force was measured with a Precisa XT 220 balance (resolution $\pm 1 \mu\text{N}$), and displacement was directly measured from the position of the crosshead (resolution $\pm 10 \mu\text{m}$), since it was calibrated that the compliance of the fiber is at least 1000 times larger than that of the experimental setup⁴⁰. Tests were performed at 0.02 mm/s^{-1} under environmental conditions 23°C and 40% RH.

The cross sectional area was measured from SEM micrographs of samples adjacent to those tensile tested. Five diameters were measured from each micrograph and the area was determined assuming a circular cross section. Samples used for observation in SEM were not subjected to supercontraction, and the cross sectional area of the samples after maximum supercontraction was calculated from the measured area assuming constant volume, as shown for *Argiope trifasciata*⁴¹. Force-displacement curves were converted to true stress-true strain curves using the calculated cross sectional areas under the constant volume hypothesis.

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Author contributions

TAB, JPR, BP and AN conducted the research. TAB and JPR wrote the main text. All authors contributed to the data analysis and reviewed the manuscript.

Additional information

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